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14. ABSTRACT

The overall goal of this project is to characterize the functioning mechanism, utility and therapeutic potential of the biomarker PRCAT47 in prostate cancer. During this first period, we investigated behind regulation of androgen receptor by PRCAT47, we developed tissue microarrays (TMAs) representing patients with clinically localized prostate cancer and metastatic castrate resistant prostate cancer, and we optimized a novel RNA in situ hybridization technology to allow us to assess PRCAT47 expression in prostate tissues. We also assessed the therapeutic potential of clinical grade anti-sense oligonucleotides (ASOs) targeting PRCAT47. ASOs were able to knock down PRCAT47 at high efficacy. Genes regulated upon ASO-mediated knockdown are highly correlated with that of siRNA-mediated knockdown. Moreover, treatment of ASOs delays prostate cancer cell growth in vitro, laying a foundation for future experiments in vivo.

15. SUBJECT TERMS

Prostate cancer; long non-coding RNA; PRCAT47

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INTRODUCTION

Prostate cancer is the second most common epithelial cancer and the second leading cause of cancer related death in men in the United States. The National Cancer Institute estimates ~180,890 new cases and ~26,120 prostate cancer related deaths in 2016 (1). Although the risk of death from indolent prostate cancer is very low, patients with aggressive and hormone-refractory form of the disease, castration-resistant prostate cancer (CRPC), frequently have poor outcomes with a median survival of only 24-30 months. Androgen Receptor (AR) and its downstream signaling play a critical role in prostate development and homeostasis as well as development and progression of prostate cancer. Androgen deprivation therapies (ADT) are front-line treatments in addition to surgery and radiotherapy for patients with high-risk localized disease, and second-generation anti-androgens such as abiraterone and enzalutamide have recently been shown to benefit patients with advanced disease. Nevertheless, CRPC is not currently curable and patients who progress to CRPC have high mortality rate. Interestingly, despite ADT, AR signaling is largely retained in CRPC though several mechanisms, including AR gene amplification, mutation and alternate splicing (2, 3). Hence, substantial efforts have been undertaken to find alternate pathways to block AR signaling. One class of tissue- and cancer- specific biomolecules that were recently identified in a variety of cancers is long non-coding RNAs (lncRNAs) that are potentially promising biomarkers and therapeutic targets (4). LncRNAs are typically spliced, polyadenylated, span over ~200 nucleotide (nt) and have been shown to play a crucial role in a variety of cellular processes. More recently, lncRNAs have also been implicated in the development and progression of a variety of cancers, including prostate cancer (5). In addition to their role in tumor initiation and progression, lncRNAs have been shown to be promising biomarkers (6). In prostate cancer, lncRNA PCA3 was shown to be a powerful prostate cancer biomarker. A PCA3-based prostate cancer diagnostic test "PROGENSA" was recently introduced. Similarly, lncRNA SCHLAP-1 was shown to be an important prognostic biomarker that can distinguish aggressive prostate cancer from indolent cancers. Despite their involvement in various cellular processes, majority of lncRNAs remain undiscovered or uncharacterized and the mechanisms through which lncRNAs act are still poorly understood. We recently identified PRCAT47, an lncRNA that is differentially expressed in localized and metastatic prostate cancer, and displayed strong transcriptional induction by AR. Preliminary data suggested that PRCAT47 has prostate tissue and cancer- specific-expression pattern, and is important for cell proliferation and regulation of AR signaling. The aim of this study is to elucidate the mechanism by which PRCAT47 regulates androgen-receptor signaling and validate it as a target for therapeutic intervention.

KEYWORDS

Prostate cancer; long non-coding RNA; PRCAT47

ACCOMPLISHMENTS

Overall Project Summary:

The overall goal of this project is to characterize the functioning mechanism and therapeutic potential of a long non-coding RNAs, PRCAT47, in prostate cancer. We hypothesized that PRCAT47 plays a critical role in regulating AR signaling and subsequently the development and progression of prostate cancer and that it may be a promising therapeutic target for treating castration-resistant prostate cancer. Three major tasks were proposed to test our hypothesis:
Specific Aim 1: delineating the molecular mechanism behind regulation of AR by PRCAT47.
Specific Aim 2: To assess clinical utility of PRCAT47 as a prognostic and diagnostic biomarker.
Specific Aim 3: Interrogating the therapeutic potential of PRCAT47 using clinical grade antisense oligos (ASOs).

Summary of Results:

During the reported research cycle, several goals were achieved under each specific aims.

Delineating the molecular mechanism behind regulation of AR by PRCAT47

In order to study how PRCAT47 regulates AR signaling, we performed AR protein and RNA stability analysis following PRCAT47 knockdown. Loss of PRCAT47 results in decreased AR stability at both protein level and RNA level. To further investigate the mechanism behind this phenotype, we examined the role of two PRCAT47-binding proteins, HuR and TIAR. We performed gene expression profiling and identified HuR/TIAR regulated gene sets in PRCAT47-psositive prostate cancer cell lines. Interestingly, within these gene sets, we observed an enrichment of AR signaling genes, indicating that these proteins are involved in AR signaling regulation. Since several PRCAT47-interacting proteins, including HuR and TIAR, are key components of stress granules, and since PRCAT47 knockdown was found to be pro-apoptotic, which is related to stress granule functions, we also studied the association of PRCAT47 with stress granules. Using RNA-in situ-hybridization, we found that PRCAT47 localizes to stress granules.

Assessing clinical utility of PRCAT47 as a prognostic and diagnostic biomarker

To interrogate the clinical potential of PRCAT47, we developed an in situ hybridization (ISH) assay for PRCAT47. In collaboration with ACD Inc., we standardized probes for PRCAT47. We subsequently evaluated the expression of PRCAT47 in a cohort of formalin-fixed paraffin-embedded (FFPE) tissues from the University of Michigan represented on tissue microarrays (TMAs).

Interrogating the therapeutic potential of PRCAT47 using clinical grade antisense oligos (ASOs)

Our preliminary data showed that PRCAT47 is required for cell growth and is highly expressed in localized and metastatic prostate cancer. We hypothesized that PRCAT47 could be utilized as a therapeutic target in prostate cancer. To test this, we assessed the therapeutic potential of clinical grade anti-sense oligonucleotides (ASOs) targeting PRCAT47. ASOs were able to knock down PRCAT47 at high efficacy. Genes regulated upon ASO-mediated knockdown are highly correlated with that of siRNA-mediated knockdown. Moreover, treatment of ASOs delays prostate cancer cell growth *in vitro,* laying a foundation for future experiments *in vivo.*

The following section will highlight the progress made in each sub-aims/ tasks proposed in the grant, including a detailed description of methods, activities, significant results, conclusion, or discussion.

1. Investigating the mechanisms of PRCAT47-mediated regulation on AR signaling using cell line models

Our previous data indicated that PRCAT47 regulates AR signaling. We also found that PRCAT47 interacts with HuR and TIAR, proteins that involved in post-transcriptional regulation of mRNA. To understand the mechanism of AR regulation, we first examined the effect of PRCAT47 knockdown on the stability of AR protein and mRNA. Then we investigated the involvement of HuR and TIAR on regulating AR signaling.

(1) Evaluation of the effect of PRCAT47 on AR protein stability.

Methods and activities:

To study whether PRCAT47 affects AR protein stability, LNCaP cells and MDA-PCa-2b cells were transfected with PRCAT47 siRNA or control RNA, then were treated with cyclohexamide, an agent that inhibits protein translation. Western blotting was performed to monitor AR protein abundance over time.

Results and conclusion:

Upon PRCAT47 knockdown, AR protein become destabilized in both LNCaP cells and MDA-PCa-2b cells, as shown in Figure 1 and 2. The result indicates that PRCAT47 regulates AR at post-transcriptional level.

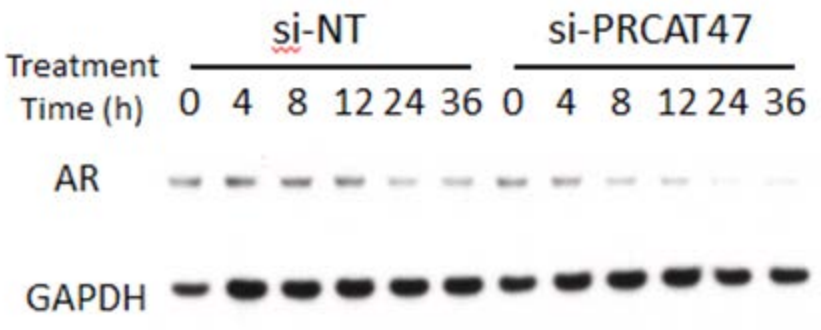

Figure 1: Western blot analysis of AR and GAPDH protein in LNCaP cells following cyclohexamide treatment for 0, 4, 8 12, 24 or 36 hours, under PRCAT47 knockdown or control condition.

Figure 2: Western blot analysis of AR and GAPDH protein in MDA-PCa-2b cells following cyclohexamide treatment for 0, 4, 8 12 or 24 hours, under PRCAT47 knockdown or control condition.

(2) Evaluation of the effect of PRCAT47 on AR mRNA stability.

Methods and activities:

To measure whether PRCAT47 affects AR mRNA stability, MDA-PCa-2b cells depleted of PRCAT47 was treated with actinomycin D, an inhibitor of mRNA transcription, and qPCR analysis was performed to quantify AR and GAPDH (Control) mRNA over time.

Results and conclusion:

Loss of PRCAT47 results in a diminished AR mRNA stability, while the stability of control RNA (GAPDH) did not change. This shortened AR mRNA half-life could in part explain the inhibited AR signaling upon PRCAT47 knockdown.

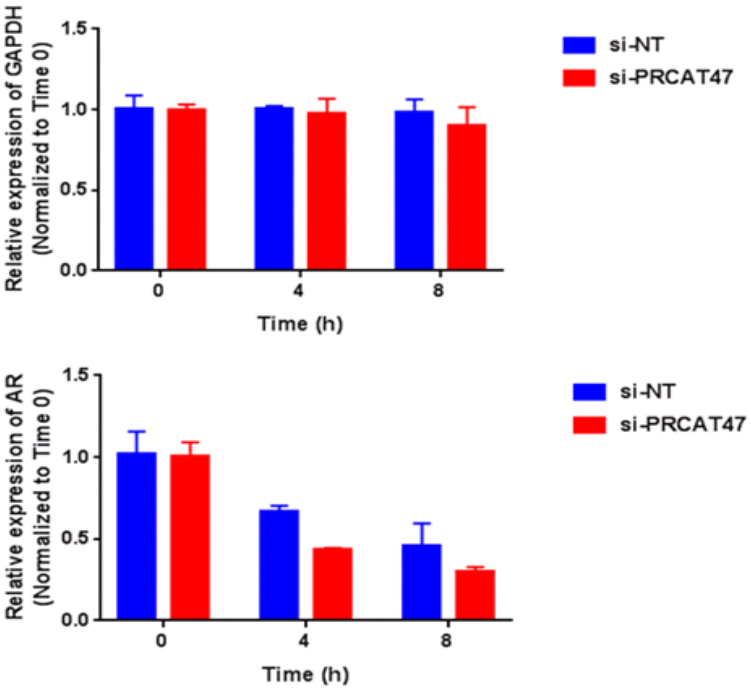

Figure 3: Relative amount of GAPDH (upper panel) and AR (down panel) mRNA changes in MDA-PCa-2b cells following actinomycin D treatment for 0, 4 and 8 hours, under PRCAT47 knockdown or control condition.

2. Understand the role of PRCAT47-interacting protein on regulating AR signaling

In order to study the functioning mechanism of PRCAT47, we previously performed RNA pull-down followed by mass spectrometry analysis to identify protein binding partners of PRCAT47. Two proteins, HuR (ELAV1) and TIAR were identified as strong binding partners of PRACT47. Given the fact that both HuR and TIAR are known to regulate the stability of messenger RNAs, including AR mRNA, we studied whether PRCAT47 functions through these two proteins, especially whether these proteins explain how PRCAT47 regulates AR signaling.

(1) Identification of HuR/TIAR regulated genes in MDA-PCa-2b and LNCaP cells.

Methods and activities:

To study the functional significance of the PRCAT47-protein interaction, we profiled the global gene expression change upon knockdown of HuR and TIAR. Microarray analysis following HuR and TIAR knockdown was performed in MDA-PCa-2b and LNCaP cells, using non-targeting siRNA as a control. Knockdown experiments were run in technical replicates, comparing knockdown samples treated with on-target siRNAs to samples treated with non-targeting control.

Total RNA from cell lines was isolated using QIAzol Lysis reagent (QIAGEN) and miRNeasy kit (QIAGEN) with DNase I digestion according to manufacturer's instructions. RNA integrity was assessed using the Agilent Bioanalyzer. Microarray analysis was carried out on the Agilent Whole Human Oligo Microarray platform, according to the manufacturer's protocol.

Results and conclusion:

HuR regulates the same set of genes between MDA-PCa-2b cells and LNCaP cells, while TIAR affects different sets of genes in two cell lines. (Figure 4) Genes with most significant changes were shown in heatmap. Interestingly, androgen receptor (AR) is one of the down-regulated genes following HuR/TIAR knockdown in both MDA-PCa-2b cells and LNCaP cells, indicating that HuR/TIAR protein is in part involved in AR signaling regulation.

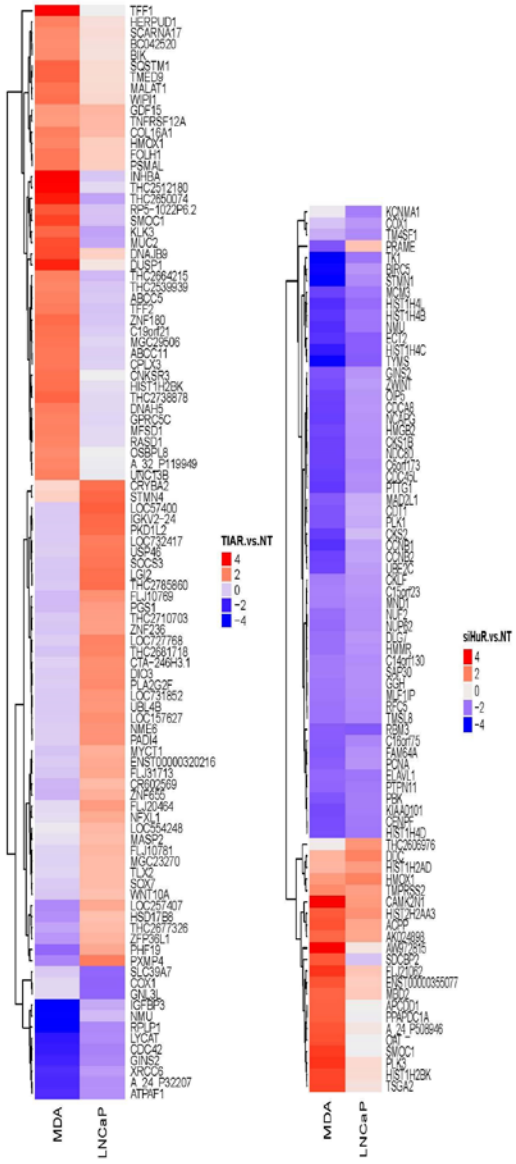

Figure 4: Gene expression profile following TIAR knockdown (Left) or HuR (Right) in MDA-PCa-2b cells and LNCaP cells. TIAR/HuR regulated genes with the most significant changes were shown in the heatmap.

(2) Studying whether AR signaling genes are regulated by HuR/TIAR

Methods and activities:

To answer the question whether genes involved in androgen receptor signaling is regulated by HuR/TIAR, we generated an AR signature using AR-positive prostate cancer cells. MDA-PCa-2b cells were treated with DHT at 10nM for eight hours (using vehicle as control). We identified genes that were significantly up-regulated or down-regulated following DHT stimulation.

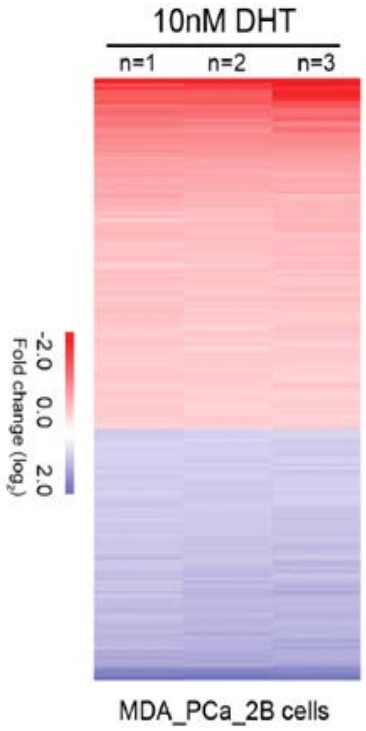

Figure 5: Reproducibility of expression profiling following 10nM DHT treatment in MDA-PCa-2b cells. The most significant AR targets were used to derive a gene signature of AR response.

Results and discussion:

Using the AR response gene signature derived above, we performed Gene Set Enrichment Analysis on HuR and TIAR regulated genes identified in (1) (**Figure 6**). TIAR loss leads to a positive enrichment of AR-inhibited genes, as well as a negative enrichment of AR-stimulated genes. Similarly, HuR knockdown results in a positive enrichment of AR-inhibited genes. The

results indicate that TIAR and HuR play a role in AR signaling regulation. It is worth exploring whether there is an overlap between PRCAT47 affected genes and TIAR/HuR target genes, and whether AR response genes are within this overlap. This goal will be achieved in the next 12 month.

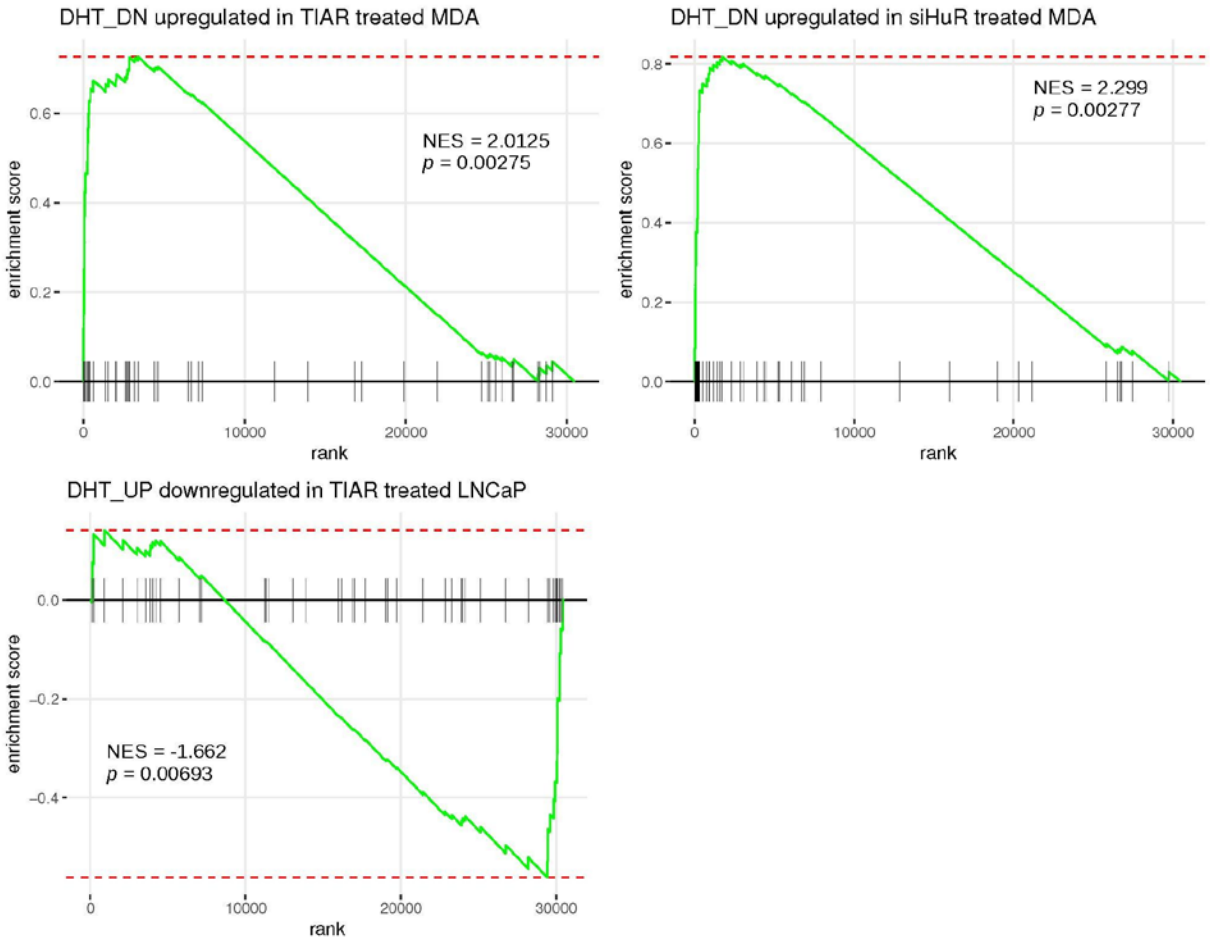

Figure 6: Gene Set Enrichment Analysis of TIAR and HuR regulated genes, using AR-response gene set. (NES represents normalized enrichment score)

3. Utilizing RIP-seq technology to study RNA-protein interaction between PRCAT47 and selected proteins and the role of PRCAT47 in global mRNA stability.

Since HuR and TIAR regulate the stability of messenger RNAs, and PRCAT47 was found to bind with these two proteins, we set out to investigate the role of PRCAT47 on global mRNA stability. This goal would be achieved in three steps: (1) Identification of the mRNA targets of both HuR and TIAR, using RNA immunoprecipitation (RIP) technology followed by high throughput sequencing. (2) Overlap of RIP-seq data with gene expression profiling generated after HuR and

TIAR KD. By this means, we would identify canonical targets of HuR and TIAR in prostate cells. (3) Overlap of PRCAT47 regulated genes with canonical targets of HuR and TIAR. This would allow us to determine if PRCAT47 is involved in HuR- or TIAR- mediated RNA stability regulation.

During this reported cycle, we performed RNA immunoprecipitation using antibodies against HuR and TIAR in MDA-PCa-2b cells and LNCaP cells, IgG was used as controls. We then performed high throughput sequencing of the bound RNA. We also profiled gene expression change after HuR and TIAR knockdown (**Figure 5**). During the next 12 month, we will perform comprehensive analysis of these data and draw conclusion on whether PRCAT47 is involved in global mRNA stability regulation.

4. Understand the role of PRCAT47 in stress granules.

We previously utilized Mass-Spectrometry and identified several PRCAT47-interacting proteins, including HuR, TIAR, YB1 and G3BP. Interestingly, these proteins are associated with stress granule formation. Given our preliminary finding that PRCAT47 knockdown induces apoptosis, and the fact that stress granule formation inhibits apoptosis, we hypothesized that PRCAT47 may localize to stress granules and contribute to stress granule formation.

Methods and activities:

To test our hypothesis, we performed immunofluorescence-based experiments to visualize the presence of PRCAT47 and stress granules. PRCAT47 was labeled by RNA fluorescence in situ hybridization (FISH), while stress granules were labeled using antibody against G3BP1 (a stress granule-associated protein). Co-localization of PRCAT47 and stress granules was monitored under normal condition and stressed condition. GAPDH, an unrelated abundant mRNA, was used as control.

Results and conclusion:

PRCAT47 localizes to stress granules in MDA-PCa-2b cells under stressed conditions. Around 25% of all PRCAT47 molecules co-localize with stress granules, which is significantly higher than non-specific co-localization between GAPDH and stress granules. This observation suggests that PRCAT47 contributes to stress granule formation.

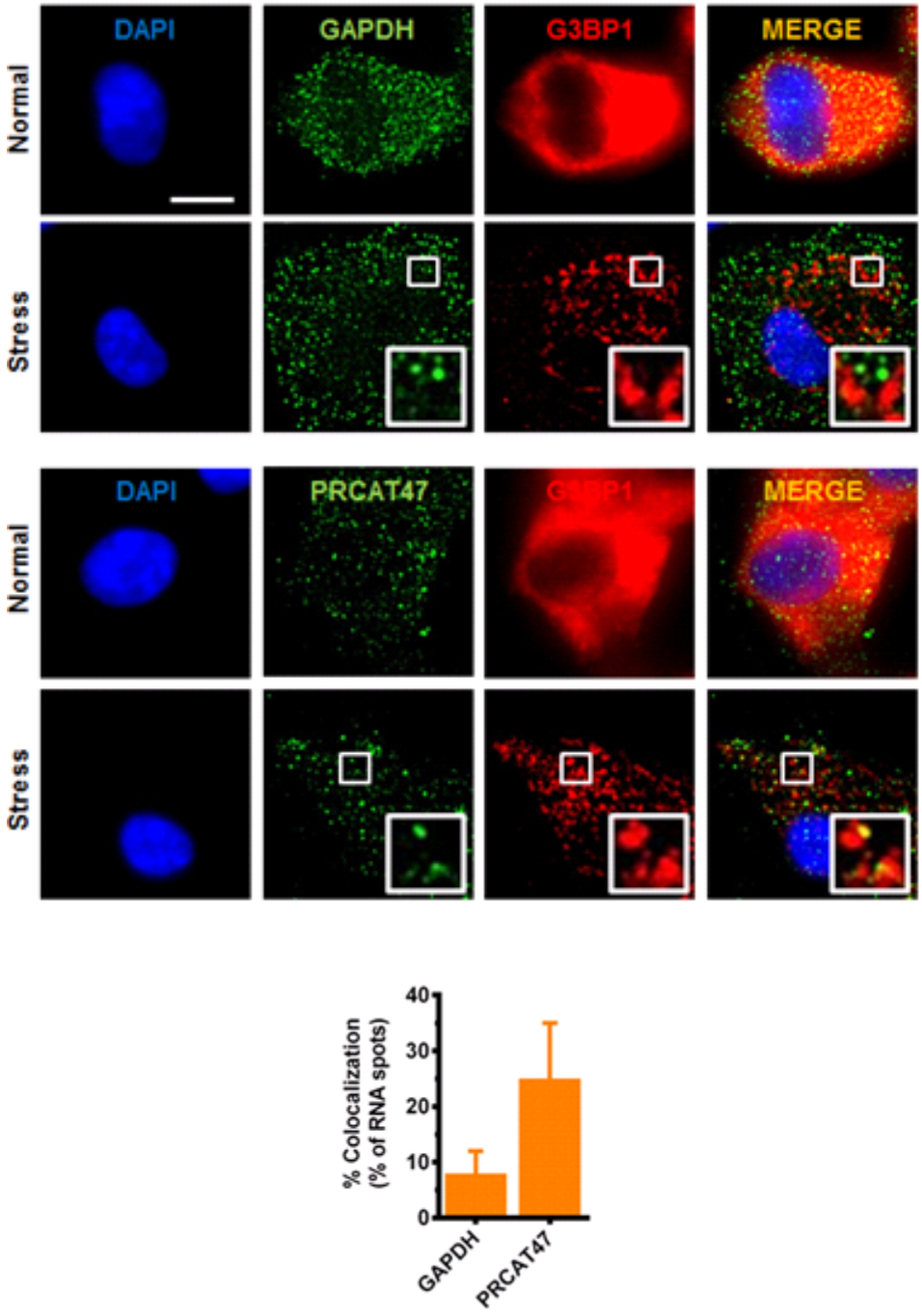

Figure 7: RNA FISH depicts co-localization of PRCAT47 transcripts and stress granules. Representative pseudo-colored images of MDA-PCa-2b cells. Cells were probed for PRCAT47 (green) or GAPDH (green, control), and G3BP1 (red). Nucleus is stained with DAPI (blue). Scale bar, 5 µm. Representative co-localized spots (G3BP1-PRCAT47) or control spots (G3BP1-GAPDH) are depicted within squares. Box plots represent the percent of RNA molecules bearing G3BP1-PRCAT47 or G3BP1-GAPDH co-localization.

5. Evaluating PRCAT47 expression in prostate cancer outcome arrays

We are in the process of performing a meta-analysis of PRCAT47 and its association with prognosis from different clinical cohorts using Affymetrix Human Exon microarray data.

6. Evaluation of PRCAT47 expression in prostate cancer tissue microarrays (TMAs) by ISH

(1) Development and standardization of probes for PRCAT47 (ACD Inc)

Methods and activities:

The RNAscope 2.0 HD BROWN assay (Cat. No. 322300) (Advanced Cell Diagnostics, Hayward, CA) was performed using target probes to on TMA slides, according to the manufacturer's instructions. RNA-ISH probe was designed and manufactured by ACD according to the specific target of interest and was ordered as single-plex probe for detection of PCAT47. Probes Hs-PPIB (human peptidylprolyl isomerase B) and DapB (bacterial dihydrodipicolinate reductase) were used as positive and negative controls, respectively.

(2) Staining, scoring and evaluating PRCAT47 expression in prostate cancer tissues with various stages.

Methods and activities:

FFPE sections were baked at 60°C for one hour. Tissues were deparaffinized by immersing in xylene twice for five minutes each with periodic agitation. The slides were immersed in 100% ethanol twice for one minute each with periodic agitation and then air-dried for five minutes. After a series of pretreatment steps, the cells were permeabilized using Protease Plus to allow probes access to the RNA target. Hybridization of the probes to the RNA targets was performed by incubation in the HybEZ™ Oven for two hours at 40°C. After two washes, the slides were processed for standard signal amplification steps. Chromogenic detection was performed using DAB followed by counterstaining with 50% Gill's Hematoxylin I (Fisher, 26801-01).

Slides were examined for PRCAT47 ISH signals in morphologically intact cells. PRCAT47 ISH signal was identified as brown, punctate dots, and expression level was scored as follows: 0 = no staining or less than 1 dot per 10 cells, 1 =1 to 3 dots per cell, 2 =4 to 9 dots per cell (few or no dot clusters), 3 =10 to 15 dots per cell (less than 10% in dot clusters), and 4 = greater than 15 dots per cell (more than 10% in dot clusters). A cumulative ISH product score was calculated for each evaluable tissue core as the sum of the individual products of the expression level (0 to 4) and percentage of cells (0 to 100) (i.e., [A%× 0] + [B%× 1] +[C%× 2] + [D% × 3] + [E% × 4]; total

range =0 to 400). For each tissue sample, the ISH product score was averaged across evaluable TMA tissue cores.

Results and conclusion:

We have performed RNA-ISH expression of PRCAT47 in a spectrum of tissues representing benign prostatic tissue, clinically localized prostate cancer (with associated long-term clinical follow-up information) and metastatic hormone refractory prostate cancer (from a unique cohort of 'warm autopsy' patients at University of Michigan). We are in the process of scoring all the cases and will compare RNA-ISH product score in different groups. For each tissue sample, the ISH product score will be averaged across evaluable TMA tissue cores. Representative images are as follows:

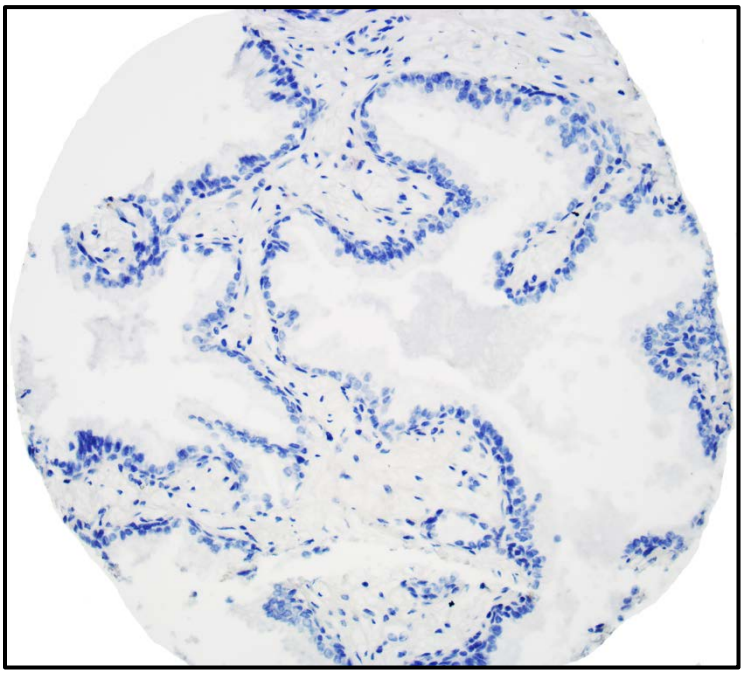

Figure 8: Representative image of benign prostate glands showing absent expression of PRCAT47

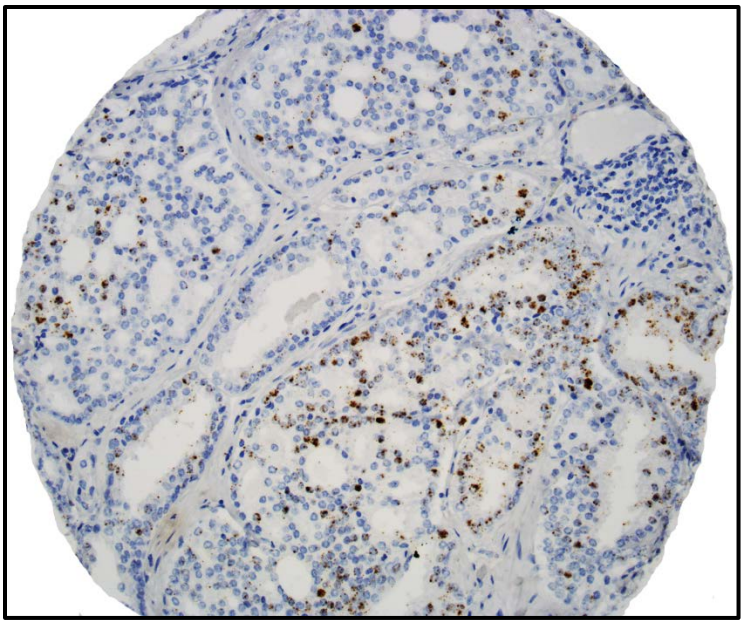

Figure 9: Representative image of localized prostate cancer showing elevated expression of PRCAT47

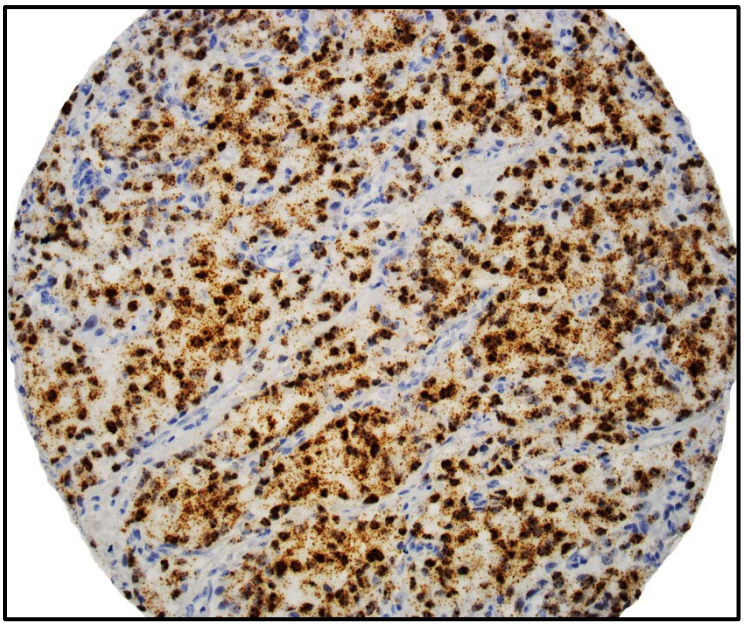

Figure 10: Representative image of metastatic prostate cancer exhibiting high expression of PRCAT47

Based on our observation, PRCAT47 expression correlates with prostate cancer progression. PRCAT47 is barely expressed in normal prostate tissue, with elevated expression in localized prostate cancer, and is highly expressed in metastatic prostate cancer.

7. Characterization of anti-sense oligonucleotides (ASOs) targeting PRCAT47 *in-vitro*.

Our preliminary data indicates that knockdown of PRCAT47 by siRNA inhibits cell proliferation and induces apoptosis. Further, cells expressing PRCAT47 shRNA generate smaller tumors in mice compared to cells that express control shRNA. Since PRCAT47 is required for cell growth and is highly expressed in localized and metastatic prostate cancer, we hypothesized that PRCAT47 could be utilized as a therapeutic target in prostate cancer.

To test this hypothesis, we started by assessing the therapeutic potential of PRCAT47 using clinical grade anti-sense oligonucleotides (ASOs) in *in-vitro* cell line models. In collaboration with Ionis pharmaceuticals, we selected six most potent ASOs targeting PRCAT47 from a pool of 80 ASOs targeting various regions of PRCAT47, based on their knockdown efficiency and off target effects. Figure 11 represents the ASO targeting sites on PRCAT47 transcript.

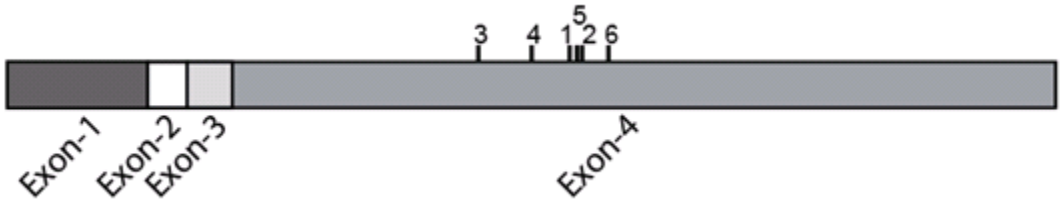

Figure 11: Diagram of antisense oligo (ASO)-targeting sites on PRCAT47 transcript.

(1) Assessment of the on-target effect of ASOs on cell lines.

Methods and activities:

To assess the on-target effect of ASOs, ASOs were delivered into cells by transfection using Lipofectamine RNAiMAX reagent according to the manufacturer's instructions. RNA was harvested for analysis 72 hours after transfection to evaluate knockdown efficiency of ASOs. cDNA was synthesized using Superscript III (Invitrogen) and random primers (Invitrogen). Relative RNA levels determined by qRT–PCR were measured on an Applied Biosystems 7900HT Real-Time PCR System, using Power SYBR Green MasterMix (Applied Biosystems). GAPDH was used as internal controls for quantification of gene targets. The relative expression of RNAs was calculated using ΔΔCt method.

Results and conclusion:

All of the six top-ranking ASOs can knockdown PRCAT47 by at least 90% 72 hours following transfection. (Figure 12)

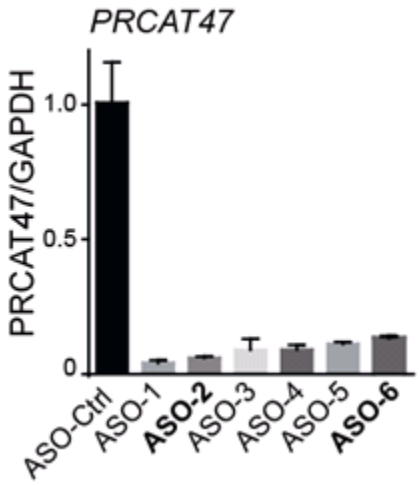

Figure 12: MDA-PCa-2b cells were transfected with six independent ASOs targeting *PRCAT47*. Knockdown efficacy was evaluated by qPCR analysis. Mean ± s.e.m are shown, n = 3.

Since PRCAT47 knockdown by siRNA led to decrease in AR mRNA and subsequent inhibition of AR target genes, we examined the changes on canonical AR targets following ASO transfection. PRCAT47 ASOs led to decreased expression of canonical AR signaling genes, including *KLK2*, *KLK3*, *FKBP5*, and *STEAP2*. This is in accordance with the gene expression change after siRNA knockdown.

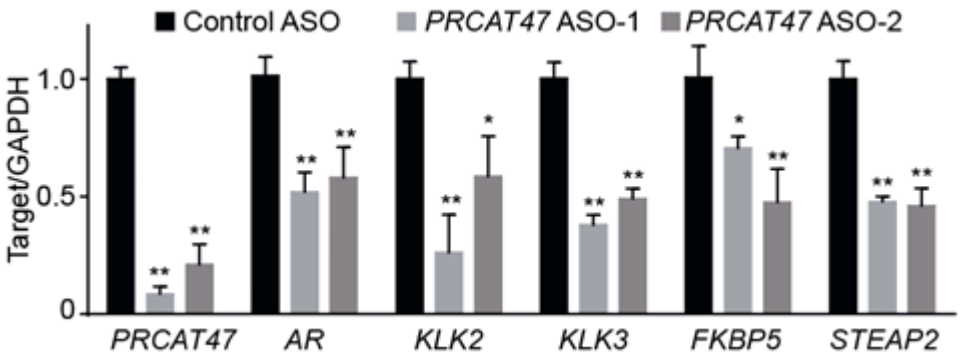

Figure 13: qRT–PCR analysis of *PRCAT47*, *AR*, and *AR* target gens (*KLK2*, *KLK3*, *FKBP5*, and *STEAP2*) in MDA-PCa-2b cells transfected with ASOs against *PRCAT47*. Data were normalized to GAPDH and the levels in control ASO-treated cells were set to 1. Mean ± s.e.m. are shown, n = 3

In conclusion, PRCAT47 ASOs induce on-target knockdown of PRCAT47, when delivered using transfection reagent. PRCAT47 inhibition by ASOs results in decreased AR signaling gene expression.

(2) Gene expression profile changes between knocking down PRCAT47 using ASOs and siRNA.

Methods and activities:

To answer the question whether knocking down PRCAT47 by two method, ASO and siRNA, results in a shared gene expression profile change, we performed Microarray analysis in MDA-PCa-2b cells, following transfection of ASO or siRNA. siRNA-mediated knockdown experiments were run in technical triplicates, comparing knockdown samples treated with two independent PRCAT47 siRNAs to samples treated with non-targeting control siRNA. ASO-mediated knockdown experiments were run in technical replicates, comparing knockdown samples treated with two PRCAT47 ASOs to samples treated with non-targeting control.

Total RNA from cell lines was isolated using QIAzol Lysis reagent (QIAGEN) and miRNeasy kit (QIAGEN) with DNase I digestion according to manufacturer's instructions. RNA integrity was assessed using the Agilent Bioanalyzer. Microarray analysis was carried out on the Agilent Whole Human Oligo Microarray platform, according to the manufacturer's protocol.

Analysis of Agilent 44k microarrays was carried out using limma and included background subtraction (bc.method="half", offset=100) and within-array normalization (method="loess"). Between array quantile normalization of average expression levels (but not log-fold changes) was performed using the function normalizeBetweenArrays (method="Aquantile"). Control probes and probes with missing values were excluded from further analyses. Probes were annotated to Gencode v22 genes using the mapping downloaded from Ensembl (efg_agilent_wholegenome_4x44k_v2). Probes originally annotated as AK093002 were used to detect PRCAT47. Differentially-expressed genes following PRCAT47 knock-down in MDA-PCA-2b cells were identified from triplicate biological repeats using adj.P.Val < 0.1 and absolute log fold-change > 0.6 cut-offs. Consensus targets of PRCAT47 knockdown using siRNA and ASOs were identified using a merged linear model (all 10 samples treated replicates) and a P value < 0.001 cut-off.

Results and conclusion:

ASO-mediated knockdown generated a gene expression profile that is similar to ASO-mediated knockdown. A common set of down-regulated genes and up-regulated genes were identified, as

shown in the heatmap in Figure 14. This includes an enrichment of canonical AR signaling genes, including *AR*, *NKX3.1*, *STEAP1*, *STEAP2*.

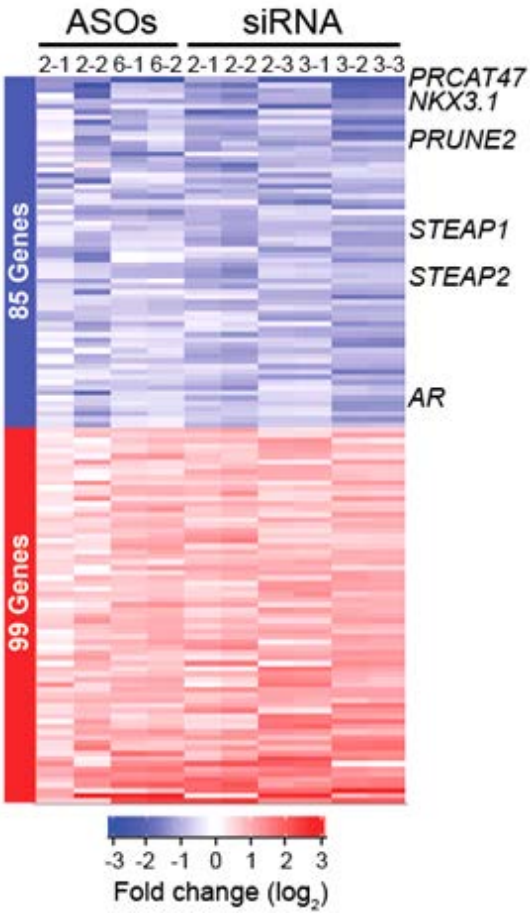

Figure 14: Gene expression profiling for siRNA-mediated or ASO-mediated *PRCAT47* knockdown in MDA-PCa-2b cells. Numbers above the heat map represent the specific siRNA / ASO and microarray replicates.

Correlation analysis was performed using differentially expressed gene sets in si-PRCAT47 condition and ASO-PRCAT47 condition. As shown in Figure 15, there exists a strong correlation between the two knockdown methods.

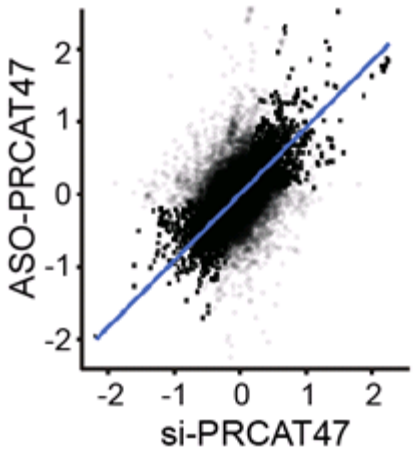

Figure 15: Correlation analysis of siRNA-mediated knockdown and ASO-mediated knockdown of PRCAT47 in replicated microarray experiments in MDA-PCa-2b cells. Each dot represents a gene. Combined significance level is indicated in shades of gray (black – most significant).

In conclusion, PRCAT47 ASO not only inhibits PRCAT47 expression efficiently, but also regulates the same set of genes compared to siRNA. This serves as a foundation for evaluating therapeutic potential of PRCAT47 using clinical grade anti-sense oligonucleotides.

(3) Assessment of the phenotypic effect and free-uptake potential of ASOs on cell lines:

In order to further select clinical-grade ASOs, Ionis pharmaceuticals performed *in-vivo* tolerability evaluation in normal Balb/c mice, using body weights, organ weights and plasma chemistry as toxicity readouts. Two of the six ASOs (ASO-2 and ASO-6) have the lowest toxicity, thus selected for characterizing their effect on cell functions.

Methods and activities:

To assess the effect of ASOs on cell proliferation, MDA-PCa-2b cells were seeded into 24-well plates in quadruplicate and allowed to attach. Cells were then transfected with ASOs and cell proliferation was recorded by IncuCyte live-cell imaging system (Essen Biosciences). PNT2 cells, which do not express PRCAT47, were used as negative control.

Since ASO free-uptake ability (uptaken by cells without lipid-based transfection reagent) is of vital importance for *in-vivo* use. We evaluated the potential of various cell lines (MDA PCa-2B, C4-2B, PNT2) to uptake ASO without any lipid-based reagent. We assessed the knockdown efficiency first and then assessed the effect of ASOs on cell proliferation.

Results and conclusion:

In MDA-PCa-2b cells, PRCAT47 ASOs (by transfection) inhibit cell proliferation, which echoes the phenotype observed using siRNA and shRNA. As a control, these ASOs failed to induce growth delay in PRCAT47-negative PNT2 cells. (Figure 16)

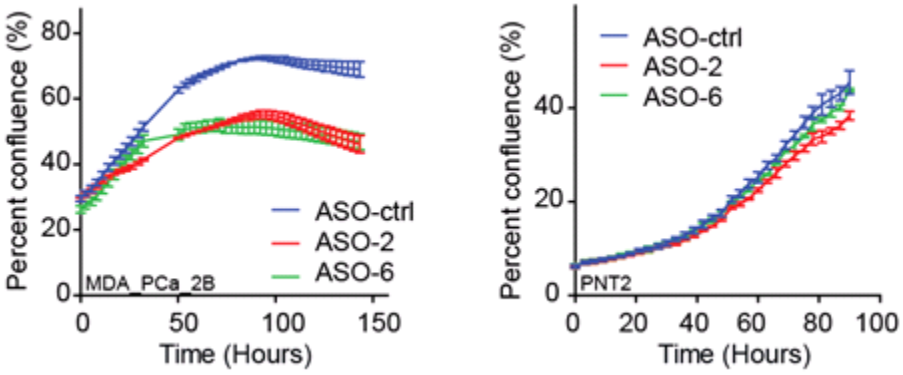

Figure 16: Transfection of ASOs targeting PRCAT47 in AR-positive MDA-PCa-2b cells inhibits cell proliferation. AR-negative prostate cell line PNT2 serves as negative control. Cell proliferation was recorded using IncuCyte live cell monitoring system.

Free-uptake efficacy of ASOs was studied in MDA-PCa-2b cells and in C4-2b cells. Although PRCAT47 ASOs were uptaken by C4-2b cells at a higher efficacy, the raw expression level of PRCAT47 is rather low in C4-2b cells. In MDA-PCa-2b cells, ASOs demonstrated difference free-uptake knockdown efficacy, ranging from 40%-80%.

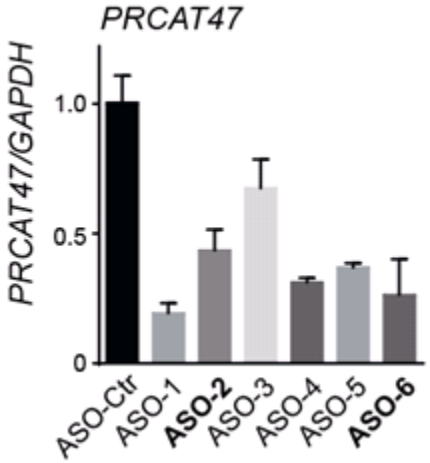

Figure 17: Free-uptake efficacy of PRCAT47 ASOs was examined in MDA-PCa-2B cells 72 hours after adding ASO to the medium (10 μM). PRCAT47 expression was evaluated by qPCR analysis. Mean ± s.e.m are shown, n = 3.

Free-uptake treatment of PRCAT47 ASOs induces growth delay of AR positive prostate cancer cells. This is validated in both single-layer cell line models (Figure 18) and 3D-sphere models (Figure 19).

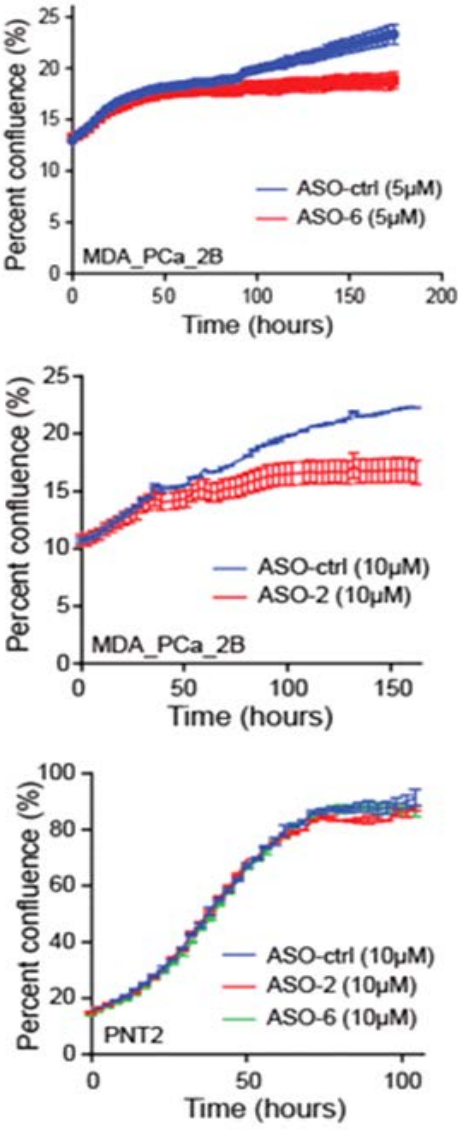

Fig 18: Treatment of ASOs targeting PRCAT47 results in retarded growth of MDA-PCa-2b cells *in vitro*. PRCAT47-negative prostate cell line PNT2 serves as negative control. Cell proliferation was recorded using IncuCyte live cell monitoring system.

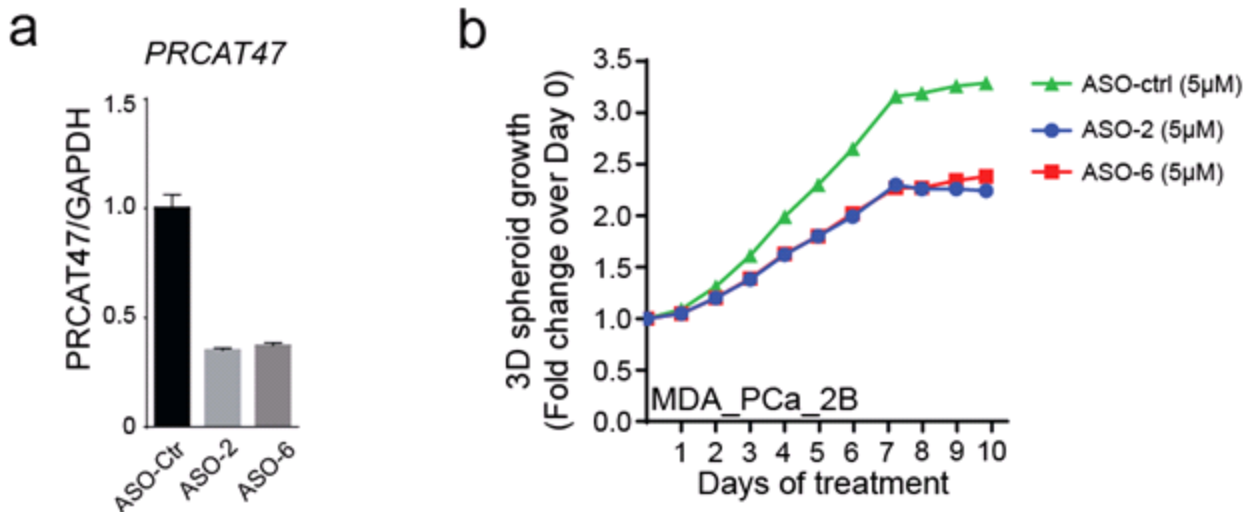

Fig 19: PRCAT47 ASOs inhibit MDA-PCa-2b cell proliferation in 3D-sphere models. Growth of 3D-spheres was recorded using IncuCyte live cell monitoring system. Cells were harvested at the end of the experiment and PRCAT47 expression was evaluated by qPCR analysis. Mean ± s.e.m are shown, n = 3.

Taken together, PRCAT47 ASOs demonstrate on-target effect and phenotypic effect *in vitro*. These ASOs not only knock down PRACT47 efficiently, but also lead to downstream gene expression changes that are similar to siRNAs. Furthermore, these ASOs could be free-uptaken by prostate cancer cell lines, serving as a foundation for future study in *in-vivo* cell line models and patient-derived xenograft models.

IMPACT

Impact on the development of the principal discipline(s) of the project:

Our preliminary data describes the discovery and characterization of a novel AR-regulated, prostate cancer- and lineage-specific long non-coding RNA, PRCAT47. We demonstrated that PRCAT47 is essential for the survival of prostate cancer cells *via* regulation of AR signaling.

In this study, we explored the functioning mechanism of PRACT47 by evaluating its effect on protein/RNA stability, probing the involvement of PRCAT47-interacting proteins. Our findings broaden the horizon of the current understanding of lncRNA functioning mechanisms.

We have successfully developed RNA-ISH probes for evaluating PRCAT47 expression from FFPE samples. We anticipate that this progress will have a significant impact on prostate cancer biology and disease management in the clinics. Data obtained as part of this proposal will aid in

the development of novel lncRNA-based biomarkers to differentiate indolent prostate cancers from aggressive disease that can be of immense value for individualized treatment in prostate cancer.

Impact on other disciplines/ technology transfer/ society beyond science and technology:

Nothing to report

CHANGES/PROBLEMS

Nothing to report

PRODUCTS

Nothing to report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	Rohit Mehra
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-6955-8884
Nearest person month worked:	1.2 CM
Contribution to Project:	Dr. Mehra has supervised proposed research and study personnel, drafted experiments, constructed tissue microarrays (TMAs) from various clinical cohorts of patients with localized and metastatic prostate cancer, performed and optimized RNA in situ Hybridization (RNA-ISH).

Name:	Xia Zhang
Project Role:	Technician
Nearest person month worked:	3.6 CM

Contribution to Project:	Ms. Zhang performed cell culture, various functional studies as well as molecular biology experiments.

Name:	Lisha Wang
Project Role:	Research fellow
Nearest person month worked:	0 CM
Contribution to Project:	Dr. Wang assisted in the development of the RNA-ISH assay.
Funding Support:	

Name:	Pankaj Vats
Project Role:	Analyst
Nearest person month worked:	1.2 CM
Contribution to Project:	Mr. Vats helped in the analysis of the high throughout data including RIP-Seq and ChIRP-seq.

SPECIAL REPORTING REQUIREMENTS

Nothing to report

APPENDICES

CRPC, castration-resistant prostate cancer
AR, Androgen Receptor
ADT, Androgen deprivation therapies
LncRNA, long non-coding RNA

References

1. Siegel RL, Miller KD, Jemal A.. CA: a cancer journal for clinicians 2016;66:7-30. CA Cancer J Clin. 2017 Jan;67(1):7-30.

2. Reid AH, Attard G, Danila DC, Oommen NB, Olmos D, Fong PC, Molife LR, Hunt J, Messiou C, Parker C, Dearnaley D, Swennenhuis JF, Terstappen LW, Lee G, Kheoh T, Molina A, Ryan CJ, Small E, Scher HI, de Bono JS. Significant and sustained antitumor activity in post-docetaxel, castration-resistant prostate cancer with the CYP17 inhibitor abiraterone acetate. J Clin Oncol. 2010 Mar 20;28(9):1489-95. doi: 10.1200/JCO.2009.24.6819. Epub 2010 Feb 16.

3. de Bono JS, Logothetis CJ, Molina A, Fizazi K, North S, Chu L, Chi KN, Jones RJ, Goodman OB Jr, Saad F, Staffurth JN, Mainwaring P, Harland S, Flaig TW, Hutson TE, Cheng T, Patterson H, Hainsworth JD, Ryan CJ, Sternberg CN, Ellard SL, Fléchon A, Saleh M, Scholz M, Efstathiou E, Zivi A, Bianchini D, Loriot Y, Chieffo N, Kheoh T, Haqq CM, Scher HI; COU-AA-301 Investigators. Abiraterone and increased survival in metastatic prostate cancer. N Engl J Med. 2011 May 26;364(21):1995-2005.

4. Iyer MK, Niknafs YS, Malik R, Singhal U, Sahu A, Hosono Y, Barrette TR, Prensner JR, Evans JR, Zhao S, Poliakov A, Cao X, Dhanasekaran SM, Wu YM, Robinson DR, Beer DG, Feng FY, Iyer HK, Chinnaiyan AM. The landscape of long noncoding RNAs in the human transcriptome. Nature genetics 2015;47:199-208.

5. Prensner JR, Iyer MK, Sahu A, Asangani IA, Cao Q, Patel L, Vergara IA, Davicioni E, Erho N, Ghadessi M, Jenkins RB, Triche TJ, Malik R, Bedenis R, McGregor N, Ma T, Chen W, Han S, Jing X, Cao X, Wang X, Chandler B, Yan W, Siddiqui J, Kunju LP, Dhanasekaran SM, Pienta KJ, Feng FY, Chinnaiyan AM. The long noncoding RNA SChLAP1 promotes aggressive prostate cancer and antagonizes the SWI/SNF complex. Nature genetics 2013;45:1392-8.

6. Mehra R, Udager AM, Ahearn TU, Cao X, Feng FY, Loda M, Petimar JS,Kantoff P, Mucci LA, Chinnaiyan AM. Overexpression of the Long Non-coding RNA SChLAP1 Independently Predicts Lethal Prostate Cancer. European Urology 2016;70:549-52.